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Changes in Synaptic Transmission Following  
Bath Application or Microinjection of  
Phospholipase A<sub>2</sub> Neurotoxins in Paired  
Cholinergic Neurons of *Aplysia* Buccal Ganglia

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13. ABSTRACT (Maximum 200 words) Taipoxin, crotoxin, and corticotxin I (C1) were tested to determine the site of action of snake neurotoxins having phospholipase A <sub>2</sub> (PLA <sub>2</sub> ) activity, using cholinergically coupled neurons of buccal ganglia of the marine mollusc <i>Aplysia californica</i> . Corticotxin II (C2), a neurotoxin that lacks PLA <sub>2</sub> activity, was also studied. The toxins were bath applied or pressure injected into presynaptic neurons. Resting membrane potentials (RMP), action potentials, and amplitude and time course of inhibitory postsynaptic potentials (IPSPs) were measured electrophysiologically. Injection or bath superfusion of all toxins usually resulted in a transient change of less than 20% in the RMP in pre- and postsynaptic cells. Injections of PLA <sub>2</sub> toxins into presynaptic neurons transiently increased IPSP amplitudes, indicating increased transmitter release. This effect was followed by time-dependent decreases in evoked IPSP amplitudes. C2 toxin, in contrast, only decreased IPSP amplitudes. Bath application of all toxins resulted in decreased IPSP amplitudes. However, bath application of toxins also resulted in increased spontaneous postsynaptic action potentials and IPSPs, suggesting effects on potassium channels. These observations suggest multiple intracellular and extracellular sites of action, in both pre- and postsynaptic neurons.				
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## Introduction

Neurotoxins with phospholipase-A<sub>2</sub> (PLA<sub>2</sub>) activity, such as  $\beta$ -bungarotoxin, crotoxin, notexin, and taipoxin, inhibit the release of ACh and produce neuromuscular blockade but, paradoxically, may induce convulsions (Brazil, 1966; Brazil and Excell, 1971; Chang and Lee, 1977; Fletcher *et al.*, 1980; Faure and Bon, 1988; Lambeau *et al.*, 1991; Lysz and Rosenberg, 1974; Rosenberg, 1986; Trivedi *et al.*, 1989; Tu, 1977). Various mechanisms for PLA<sub>2</sub> neurotoxicity have been proposed, including digestion of internal or external cellular membranes, byproduct insertion into membranes resulting in a disruption of secretory vesicles (Rosenberg, 1975; Tu, 1977), compromise of mitochondria and electron transport chains (Lin and Lee, 1974; Tu, 1977), blockade of ion channels (Dreyer, 1990; Strong, 1990) or an indirect interference with transmitter release caused by an accumulation of products of enzymatic digestion (Tu, 1977) or inhibition of protein phosphorylation (Ueno and Rosenberg, 1990; 1992).

In a series of experiments to define the site of intoxication, Simpson *et al.* (1993), using rat hemidiaphragms, reported that both crotoxin and taipoxin induced a concentration-dependent blockade of neuromuscular transmission. Two possible mechanisms were suggested.

1. The toxins act on an external or transmembrane site, possibly a voltage-dependent potassium channel. This is in agreement with studies reported earlier by Dreyer (1990) and Strong (1990), who concluded that crotoxin and other PLA<sub>2</sub> neurotoxins may bind to voltage-dependent potassium channels. This effect may be independent of phospholipid hydrolysis (Dreyer and Penner, 1987; Rosenberg, 1986; Rowen and Harvey, 1988; Ueno and Rosenberg, 1990).
2. The toxins are internalized and then act upon an internal target. Experiments using neutralizing antibodies indicated that the PLA<sub>2</sub> neurotoxins associate with binding sites on or near the cell surface, and then may become internalized or sequestered within the membrane. Additional studies led Simpson *et al.* (1993) to conclude that internalization of PLA<sub>2</sub> neurotoxins is not necessary to produce blockade, nor is binding itself the toxic step. Thus, Simpson *et al.* (1993) concluded that there are still uncertainties as to whether intoxication is an extracellular or intracellular event. The purpose of this study was to attempt, by either bath-applying or intracellularly injecting the toxins, to determine whether their site of action is extracellular or intracellular.

## Materials and Methods

### *Electrophysiology*

To determine the site of action of snake neurotoxins having PLA<sub>2</sub> activity, toxins were delivered by intracellular injection (intracellular application) or by bath superfusion (extracellular application) and compared for their effects on synaptically coupled cholinergic neuron pairs in isolated buccal ganglia of the marine mollusc *Aplysia californica*. Inhibition of nerve-evoked release of acetylcholine (ACh) was measured at identified cholinergic synapses in buccal ganglia (Gardner, 1971) using procedures similar to those described by Poulain *et al.* (1988). The buccal ganglia were surgically removed and pinned to the Sylgard® (Dow Corning, Midland, MI) lined bottom of an acrylic chamber, and the connective tissue capsule was removed using

microsurgical techniques. The soma of identified pre- and postsynaptic cholinergic neurons (Fig. 1) were impaled with glass microelectrodes (2-4 M $\Omega$ ) filled with 2 M potassium acetate. Action potentials were evoked in presynaptic neurons by suprathreshold depolarizing stimuli applied once per 10 sec. Neurotransmitter release was assessed by measuring the amplitude of the inhibitory postsynaptic potential (IPSP) recorded in a voltage-clamped follower neuron. Presynaptic and postsynaptic potentials were digitized and stored on a laboratory computer using pClamp software (Axon Instruments, Inc., Foster City, CA). Responses were analyzed only if they were not obscured by spontaneous action potentials or IPSPs, which were frequent in some preparations in the presence of toxins. The preparation was superfused continuously at a rate of 1 ml/min with artificial sea water (ASW). The ganglia were maintained at room temperature during the experiments.

Cholinergic cell pairs were identified on the basis of location, electrophysiological characteristics, and response to pharmacological agents (Gardner and Kandel, 1977). Presynaptic neurons were those cells designated by Gardner (1971) and Gardner and Kandel (1977) as B4 and B5, and postsynaptic neurons were cells B3, B6, B8, B9 and B10 (Fig. 1). Postsynaptic cells were hyperpolarized by focal extracellular application via pressure ejection from a micropipette of acetylcholine (ACh) ( $10^{-4}$  M) or the nonhydrolyzable ACh agonist carbachol ( $10^{-4}$  M). Evoked IPSPs were blocked by the nicotinic cholinergic antagonist d-tubocurarine ( $10^{-4}$  M), and exaggerated by the cholinesterase inhibitor edrophonium ( $10^{-4}$  M). Changes were quantified in the pre- and postsynaptic resting membrane potentials (RMP), in the amplitude, duration, and waveform of the presynaptic evoked action potential, and in the IPSP amplitude and duration.

### Toxins

Crotoxin and taipoxin were supplied by J.L. Middlebrook. Crotoxin, from *Crotalus durissus terrificus*, was isolated and purified by standard methods (Aird and Kaiser, 1985; Middlebrook and Kaiser, 1989). Taipoxin, from the venom of *Oxyuranus scutellatus*, was isolated and purified as described by Fohlman *et al.* (1976), as modified by Middlebrook and Kaiser (1989). Purities of these toxins were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The mouse LD<sub>50</sub> of taipoxin was 2  $\mu$ g/Kg, and 50  $\mu$ g/Kg for crotoxin (Middlebrook and Kaiser, 1989). Corticotoxin I (C1) is a 20 kD toxin that can suppress EEG activity. Corticotoxin II (C2) is a 5 kD neurotoxin that produces physiological effects similar to C1, but lacks PLA<sub>2</sub> activity. C1 and C2 were supplied by C.A. Broomfield. C1 and C2 were isolated, purified and crystallized from venom of the Indian cobra, *Naja naja*, as described by Currie *et al.* (1968).

Toxins were delivered externally by addition to a 2-ml bath containing ASW. Fluid flow, which was usually 1 ml/min, was stopped while toxin was present in the bath. Excess toxins were washed out with ASW after 20 minutes. Toxins delivered intracellularly were pressure injected as a bolus into the presynaptic neuron. Crotoxin was superfused or injected at a concentration of  $3.5 \times 10^{-5}$  M, taipoxin was superfused at a concentration of  $5 \times 10^{-6}$  M or pressure injected at  $10^{-6}$  M, and C1 and C2 were superfused or injected in  $10^{-6}$  M solutions. Selection of these concentrations was based on solubility and availability of the toxins.

## *Media*

Lyophilized toxins were rehydrated with distilled water prior to suspension in ASW. A commercial dye, FD & C Blue No. 1, at a concentration of 1:8 in ASW, was used to aid in the visualization of toxin injection. Ejection pressure was 60 PSI, applied for sufficient time to inject an adequate volume of toxin. The amount of material delivered by micropipette was visually judged to be <10% of the cell volume on the basis of the color of the dye mixed with the toxins. Sufficient amounts of toxin-dye mixture were injected to yield a consistent final color in each experiment. The composition of ASW was (mM) NaCl 480; KCl 10; CaCl<sub>2</sub> 10; MgCl<sub>2</sub> 20; MgSO<sub>4</sub> 30; NaHCO<sub>3</sub> 2.5; pH 7.7. The connective tissue capsule was dissected from the buccal ganglia in hypertonic ASW to minimize neuronal damage. The dissection solution was made hypertonic (1500 mOsm/kg) by the addition of 13.7 g sucrose per 100 ml ASW.

## *Controls*

Injections of dye alone or dye mixed with bovine serum albumin (BSA) were used as controls to test for nonspecific protein effects. Other drugs used included arachidonic acid, bath applied [100  $\mu$ M] only. A minimum of three preparations were used for both extracellular and intracellular application of each drug or toxin.

## **Results and Discussion**

The results of these experiments fall within three general categories:

### *1. Effects on RMP and action potentials*

Application of PLA<sub>2</sub> toxins had no remarkable or consistent effect on the amplitude of the presynaptic action potential (Fig. 2) or on the RMP in either presynaptic or postsynaptic cells, whether superfused or injected into the presynaptic cell (Fig. 3). The resting membrane potential (RMP) and the amplitude of the presynaptic action potential reflect the integrity of cellular membranes and of the processes that maintain membrane function. Membrane disruption usually results in decreased RMP. One proposed mechanism for the PLA<sub>2</sub> toxin-related inhibition of ACh release has been the direct enzymatic digestion of cellular membranes (Rosenberg and Condrea, 1968; Rosenberg, 1975; Tu, 1977) and/or disruption of mitochondria and electron transport chains (Lin and Lee, 1974; Tu, 1977).

Our results suggest that these toxins do not have nonspecific effects on passive membrane properties. Based on changes in the evoked IPSP, transmitter release was eventually reduced (see Fig. 4). This decreased transmitter release did not appear to depend on changes in RMP.

### *2. Effects on neurotransmitter release*

Changes in neurotransmitter release can be inferred from changes that occur in the amplitude of the IPSP. Superfusion of all toxins (Fig. 4a) caused a decrease in the IPSP amplitudes at 40



min. The toxin that caused the largest measurable decrease in IPSP amplitude (56%) at 60 min was C1. IPSP amplitudes were also decreased by 14% (probably not significant) by crotoxin and taipoxin. However, all toxins, especially C1 and, to a lesser extent, crotoxin, taipoxin, and C2, caused a dramatic increase in spontaneous activity following bath application (see Fig. 5). This hyperactivity made it difficult to measure IPSP amplitudes accurately, and, in the case of the non-PLA<sub>2</sub> C2 toxin, evoked IPSPs were totally obscured by spontaneous activity within 5 min (Fig. 4a). In general, injection of the PLA<sub>2</sub> neurotoxins (Fig. 4b) caused an initial increase in ACh release as indicated by an increase in the evoked IPSP amplitude and duration, followed by a decreased IPSP amplitude and duration. Injection of any substance (BSA, buffers) tends to cause this transient, probably nonspecific increase in IPSP amplitude, which generally returns to normal within 30 min. In contrast, and unexpectedly, only two of six preparations receiving the non-PLA<sub>2</sub> toxin, C2, showed the transient increase in evoked IPSP amplitudes. The inhibitory action of the C2 toxin may have overcome the transient IPSP amplitude increase normally seen. Within 10 min after C2 injection, IPSP amplitude had decreased by 60% and remained depressed by 45% at 40 min. By 30 min, crotoxin caused a 23% decrease in IPSP amplitudes, and C1 caused a similar decrease (18%) at 40 min. Taipoxin alone did not cause a net decrease in IPSP amplitudes. This result may have been due to inadequate injection. The loss of response to the evoked action potential did not appear to be due to decreased sensitivity to ACh, because postsynaptic cells responded normally to addition of ACh to the bath and to micro-application of ACh directly onto the neurons.

The initial increase in IPSP amplitude could be due to a mechanism involving PLA<sub>2</sub>-induced release of arachidonic acid (AA) and related metabolites. PLA<sub>2</sub> enzymes act on internal and external cellular membranes to catalyze the release of free fatty acids and liberate AA. PLA<sub>2</sub>-induced AA release has been implicated in secretory events, including the enhancement of calcium-dependent stimulation of ACh release from neurons and/or a build up of related metabolites (Ray *et al.*, 1993; Tu, 1977; Volterra *et al.*, 1992). For instance, crotoxin has been reported to enhance the secretion of substances other than neurotransmitters (Ollivier-Bosquet *et al.*, 1991). When crotoxin was internalized into epithelial mammary cells, it increased secretion of casein in a manner comparable to prolactin, which acts through the liberation of AA from phospholipids (Ollivier-Bosquet *et al.*, 1991). Similarly, other PLA<sub>2</sub> enzymatic products, including lysolecithin (lysophosphatides), have been implicated in increased secretion and increased membrane permeability (Tu, 1977). A lysolecithin-dependent toxigenesis has been proposed in which increased levels of membrane lysolecithin produce weakening and breakdown of synaptic vesicles. Vesicle-bound ACh is released as a consequence of this breakdown. Hypersecretion is followed by axonal blockade as vesicles become unavailable (Tu, 1977; Heilbronn, 1972).

We found that AA superfusion, in the absence of toxins, caused a small increase in IPSP amplitudes; however, AA did not produce the later postsynaptic amplitude decreases (indicating decreased transmitter release) nor cause spontaneous activity increases indicating potassium channel blockade (data not shown). Therefore, not all of the cellular effects of PLA<sub>2</sub> toxins could be accounted for by production of AA or related metabolites.

Decrease of ACh release occurred in neurotoxin-injected cells, as evidenced by the decline of IPSP amplitude in the postsynaptic cells. This IPSP amplitude decrease did not usually occur if

the externally applied toxin was washed out before 20 minutes of application (data not shown).

Thus, unless the toxin effects are extremely slow in onset, an internal mechanism appears to be involved in transmitter failure.

An alternative mechanism for inhibition of transmitter release, which does not involve actions on  $K^+$  channels, has been proposed for  $\beta$ -bungarotoxin. This toxin may act intracellularly, inhibiting the phosphorylation of synapsin I and other synaptosomal proteins involved in secretion (Ueno and Rosenberg, 1990; 1992).

### 3. *Effects on spontaneous activity*

Superfusion of AA, crotoxin, taipoxin, C1 and C2, resulted in increased spontaneous activity, with increased frequencies of action potentials and IPSPs, in both presynaptic and postsynaptic cells (Fig. 5). Each toxin produced its own characteristic waveform pattern. The non-PLA<sub>2</sub> toxin, C2, caused the most dramatic change, with almost continuous activity coupled with cyclic increases and decreases in the RMP. C1 caused the most irregular activity. Taipoxin typically caused regular spontaneous IPSPs between evoked ones, and crotoxin caused regular spontaneous IPSPs and occasional transient changes in RMP. The increase in spontaneous activity may be due to toxin-induced blockade of voltage-dependent potassium channels (Dreyer, 1990; Strong, 1990).

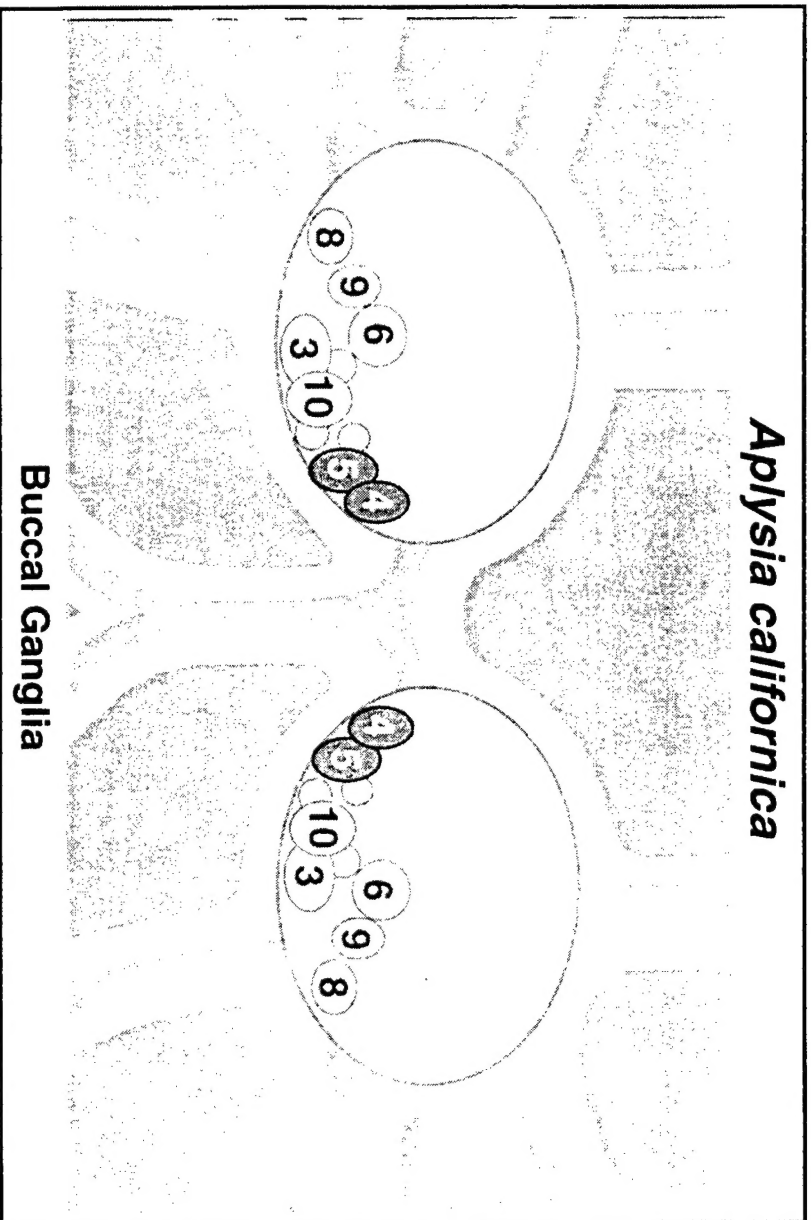
## Conclusions

In the *Aplysia* model, PLA<sub>2</sub> neurotoxins induced changes in both the pre- and postsynaptic neurons when delivered by either injection or bath superfusion. Considering the enzymatic nature of PLA<sub>2</sub> neurotoxins, surprisingly few direct membrane changes were detected. Changes in the evoked IPSP, following injection of PLA<sub>2</sub> toxins into presynaptic neurons, indicated a change in neurotransmitter release in two stages: initially a nonspecific increase, followed by a delayed inhibition of ACh release as determined by decreased IPSP amplitude. C2 toxin usually caused only a decrease in IPSP amplitudes.

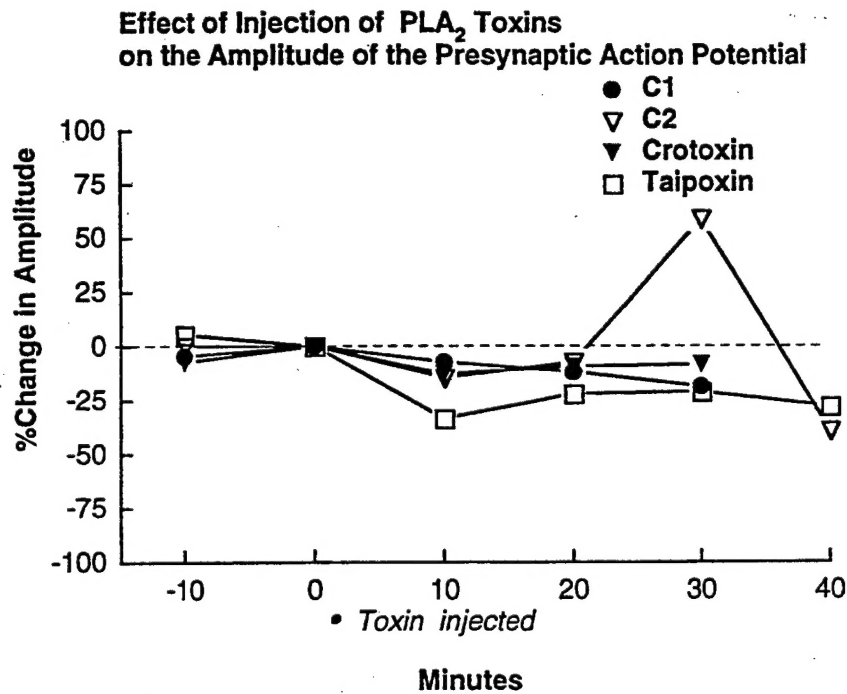
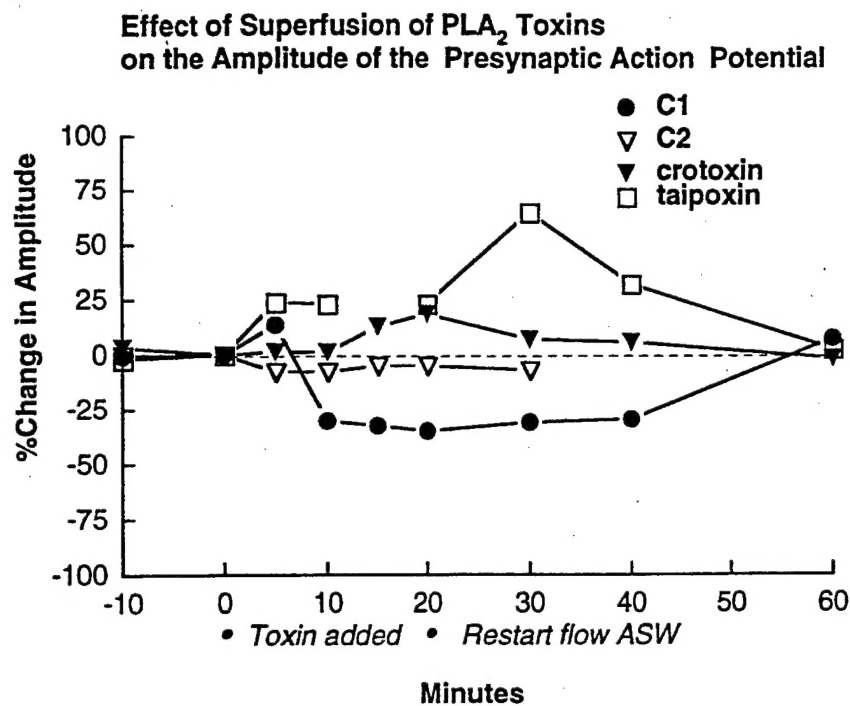
Effects of external application of all toxins included small reversible changes in the RMP in both pre- and postsynaptic cells, and also a time dependent decrease in release of ACh as indicated by a decreased amplitude and duration in the evoked IPSP. Recovery occurred if toxins were washed out within 20 minutes of application. Superfusion of toxins also resulted in greatly increased spontaneous postsynaptic activity.

These observations may explain some of the controversial and seemingly paradoxical reports in the literature regarding whether PLA<sub>2</sub> neurotoxins act intracellularly or extracellularly. Our results suggest that these toxins produce complex effects by interacting with both intracellular and extracellular sites in both pre- and postsynaptic neurons.



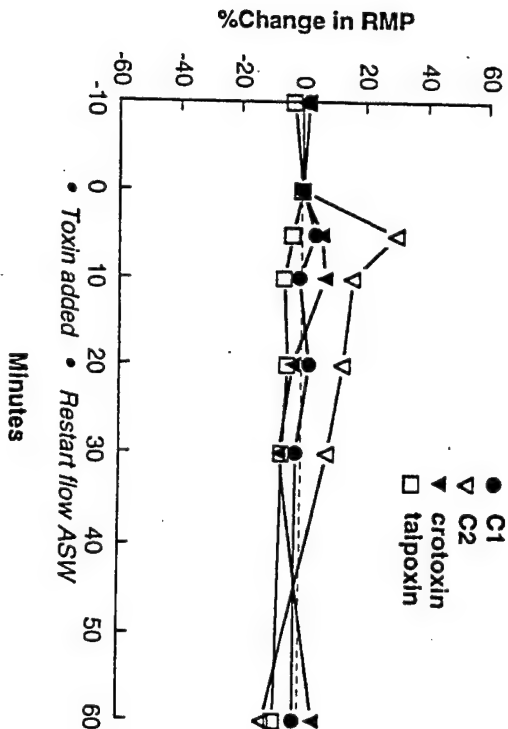


**Figure 1. Schematic diagram of the cephalic surface of the paired buccal ganglia.** Numbered presynaptic (dark) and postsynaptic (light) shaded cells are indicated in left and right hemiganglia. Each presynaptic neuron in each hemiganglion projects to all of the indicated postsynaptic neurons within the same hemiganglion. Presynaptic neurons do not project to the opposite side. (Modified from Gardner and Kandel, 1977).

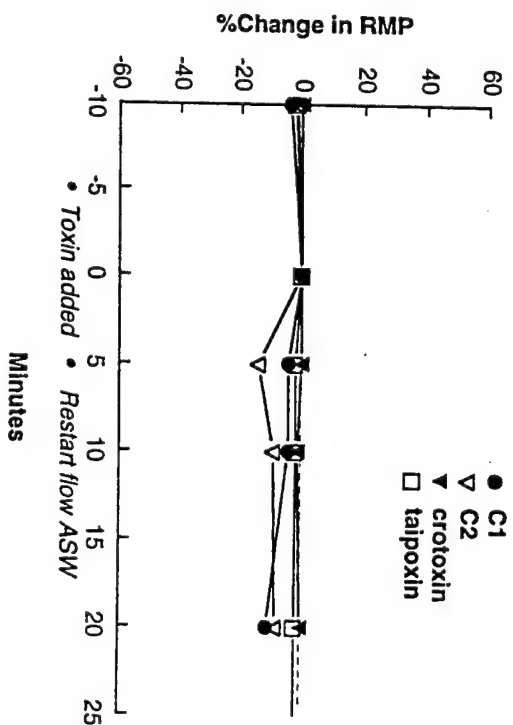


**Figure 2. Effect of neurotoxins on the amplitude of the presynaptic action potential.** Results of a representative experiment. A. Superfusion of the toxins had no significant effect. The increase in amplitude in the presence of taipoxin after restarting flow of ASW is a frequent consequence of restarting flow after a period of stasis. B. Injection of toxins likewise had no significant effect. The increase in amplitude at the 30-min time point with C2 toxin was transient and probably artifactual.

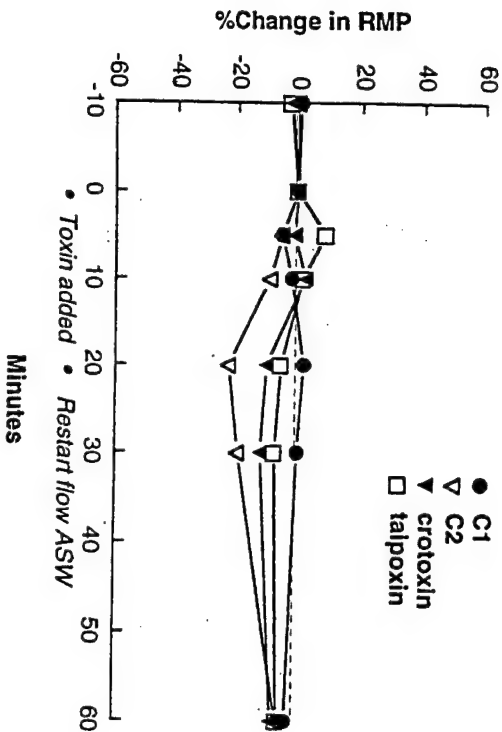
**Effect of Superfusion of Phospholipase A2 Neurotoxins on Presynaptic Resting Membrane Potential**



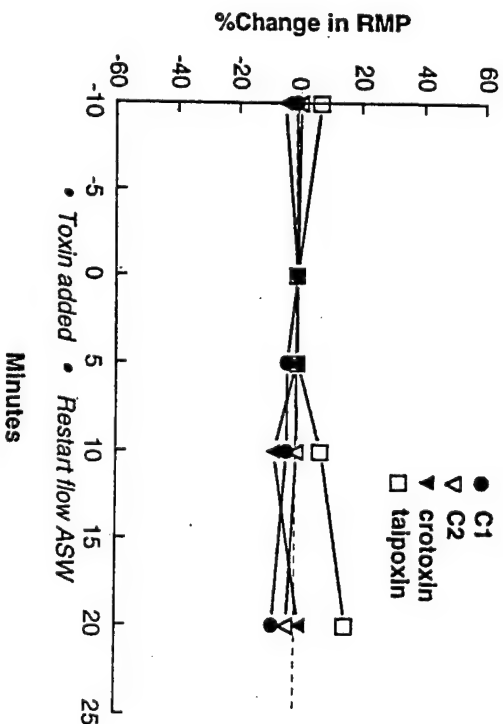
**Effect of Injection of Phospholipase A2 Neurotoxins on Presynaptic Resting Membrane Potential**



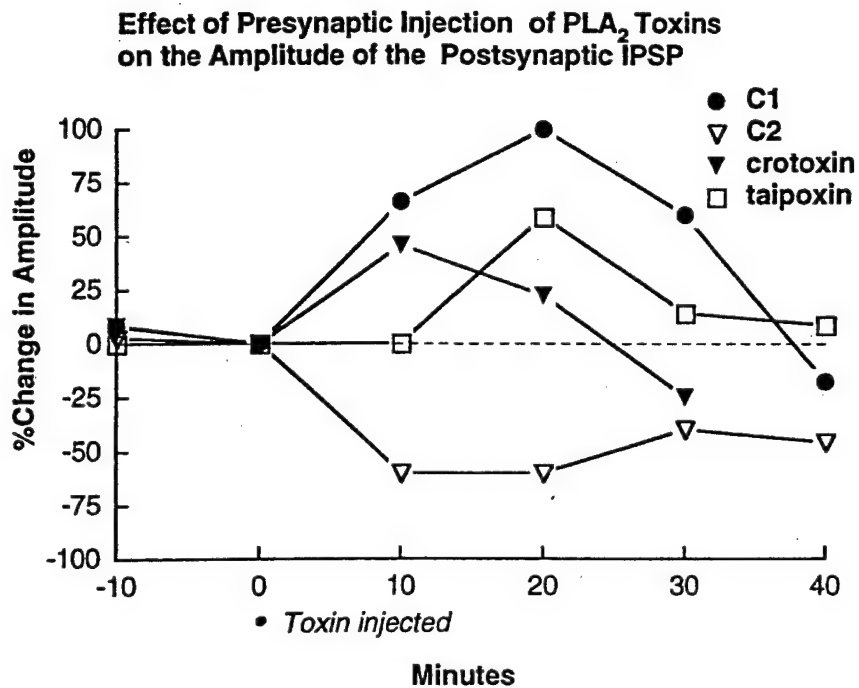
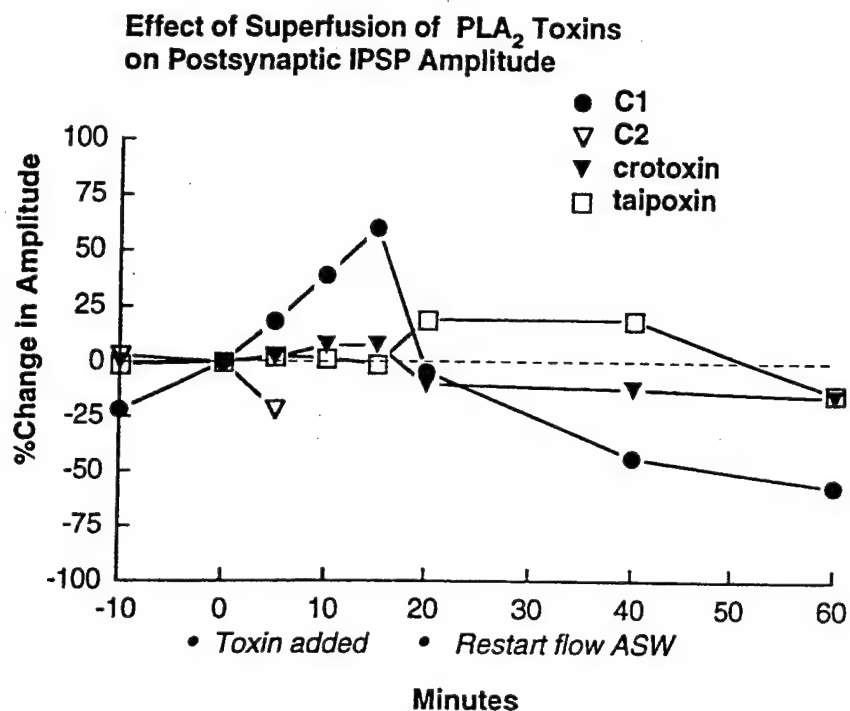
**Effect of Superfusion of Phospholipase A2 Neurotoxins on Postsynaptic Resting Membrane Potential**



**Effect of Injection of Phospholipase A2 Neurotoxins on Postsynaptic Resting Membrane Potential**



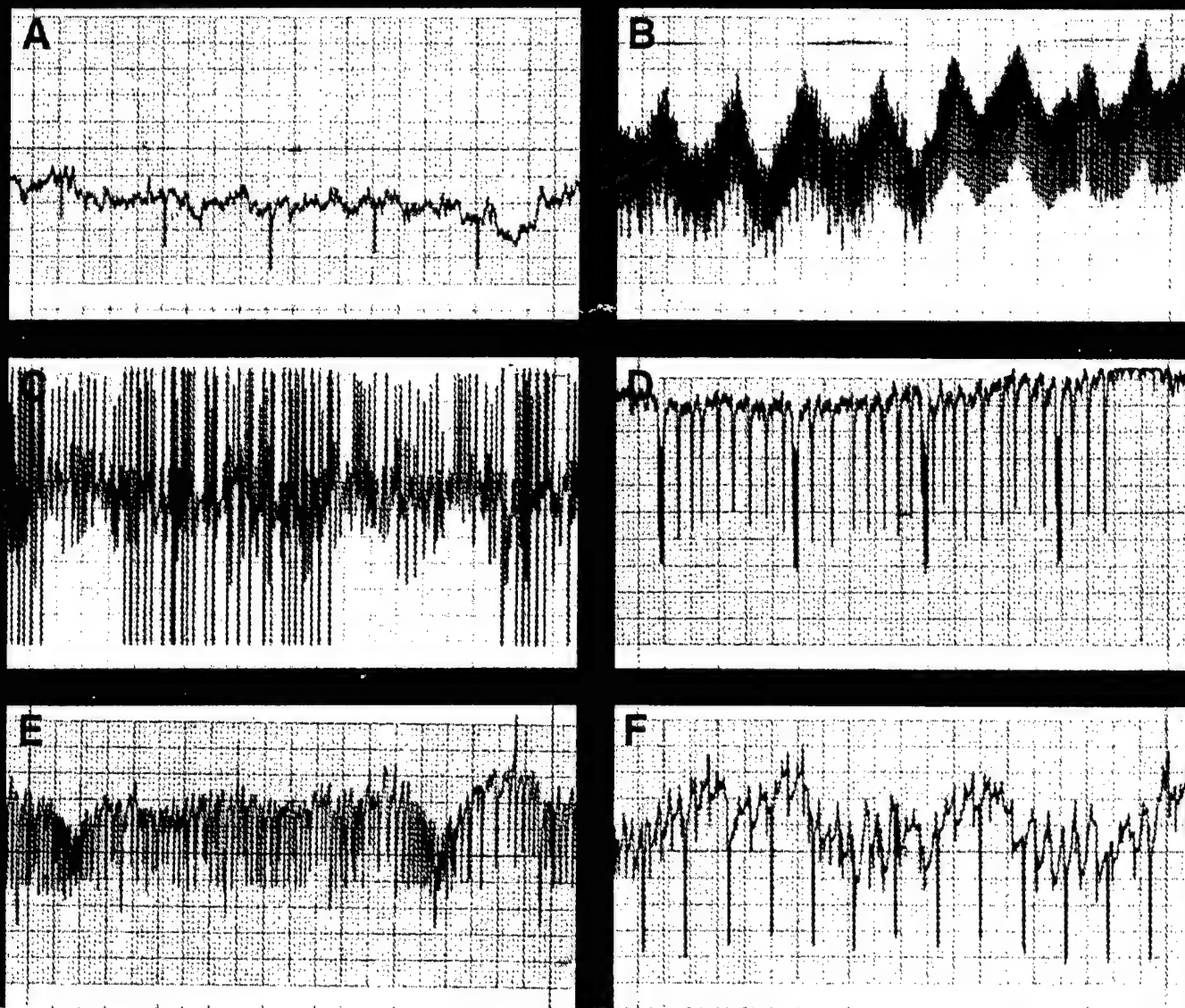
**Figure 3. Effect of PLA<sub>2</sub> toxins on presynaptic and postsynaptic resting membrane potentials. Results of a representative experiment. Superfusion of toxins had no sustained effects on the presynaptic (A) or postsynaptic (C) resting membrane potentials. Injection of toxins had no effect on the presynaptic (B) or postsynaptic (D) resting membrane potentials.**



**Figure 4. Effect of PLA<sub>2</sub> toxins on IPSP amplitudes.**

Results of a representative experiment. A. Superfusion of C1 toxin caused a large decrease in IPSP amplitude. Crotoxin and taipoxin caused smaller decreases that are probably not meaningful. IPSPs following superfusion of C2 toxin were obscured by intense spontaneous activity in pre- and postsynaptic neurons after 5 min, so no data points were available thereafter. B. Injection of all toxins except taipoxin caused a decrease in IPSP amplitude by 40 min postinjection. The volume of injection of taipoxin may have been inadequate.

## Post-synaptic Spontaneous Activity



**Figure 5.** Superfusion of PLA<sub>2</sub> toxins caused intense spontaneous activity in addition to evoked responses in neurons, as reflected by recordings from representative postsynaptic cells. A. Control IPSPs. B. C2 bath effect on IPSPs 10 min post-injection. C. C1 bath effect on IPSPs after 15 min. D. Taipoxin bath effect on IPSP after 15 min. E. Crotoxin bath effect on IPSP after 20 min. F. Recovery of IPSPs after washout of crotoxin.

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